



#7

2570-1-001N

## HIV-1 VACCINES AND SCREENING METHODS THEREFOR

### CROSS-REFERENCE TO RELATED APPLICATION

Priority under 35 U.S.C. §119(e) is claimed to Provisional Application Serial No. 60/214,608, filed June 27, 2000, and which is incorporated herein by reference in its entirety.

### RESEARCH SUPPORT

The research leading to the present invention was supported in part by the Public Health Service, National Institutes of Health grants AI47708-01 and AI 44309-01. The government may have certain rights in the present invention.

### BACKGROUND OF THE INVENTION

DNA immunization stimulates both the cellular and humoral arms of the immune system (Liu, M. A., Y. Yasutomi, M.-E. Davis, H. C. Perry, D. C. Freed, N. L. Letvin, and J. W. Shiver. 1996. Vaccination of mice and nonhuman primates using HIV-gene-gun-containing DNA, vol. 48. Karger, S, Basel; Shiver, J. W., M.-E. Davies, H. C. Perry, D. C. Freed, and M. A. Liu. 1996. Humoral and cellular immunities elicited by HIV-1 DNA vaccination. J. Pharm. Sci. 85:1317-1324; Shiver, J. W., H. C. Perry, M.-E. Davies, D. C. Freed, and M. A. Liu. 1995. Cytotoxic T lymphocyte and helper T cell responses following HIV polynucleotide vaccination. DNA Vaccines. 772:198-208; Shiver, J. W., J. B. Ulmer, J. J. Donnelly, and M. A. Liu. 1996. Humoral and cellular immunities elicited by DNA vaccines: Application to the human immunodeficiency virus and influenza. Adv. Drug Del. Rev. 21:19-31-18) and elicits

1 immune responses capable of preventing infection of animals by slowly replicating  
 2 viruses, such as HIV-1 in chimpanzees (Boyer, J. D., K. E. Ugen, B. Wang, M.  
 3 Agadjanyan, L. Gilbet, M. L. Bagarazzi, M. Chattergoon, P. Frost, A. Javadian, W. V.  
 4 Williams, Y. Refaeli, R. B. Ciccarelli, D. McCallus, L. Coney, and D. B. Weiner.  
 5 1997. Protection of chimpanzees from high-dose heterologous HIV-1 challenge by  
 6 DNA vaccination. *Nature Med.* 3:526-532). However, when the challenge virus  
 7 replicates efficiently in the host, such as SIV or SHIV in macaques, the DNA-elicited  
 8 immune responses offer only partial protection (Boyer, J. D., B. Wang, K. E. Ugen, M.  
 9 Agadjanyan, A. Javadian, P. Frost, K. Dang, R. A. Carrano, R. Ciccarelli, L. Coney,  
 10 W. V. Williams, and D. B. Weiner. 1996. In vivo protective anti-HIV immune  
 11 responses in non-human primates through DNA immunization. *J. Med. Primatol.*  
 12 25:242-250; Lu, S., J. Arthos, D. C. Montefiori, Y. Yasutomi, K. Manson, F. Mustafa,  
 13 E. Johnson, J. C. Santoro, J. Wissink, J. I. Mullins, J. R. Haynes, N. L. Letvin, M.  
 14 Wyand, and H. L. Robinson. 1996. Simian immunodeficiency virus DNA vaccine trial  
 15 in macaques. *J. Virol.* 70:3978-3991; Robinson, H. L., D. C. Montefiori, R. P.  
 16 Johnson, K. H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P.  
 17 Mazzara, D. L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M.  
 18 S. Wyand, and H. M. McClure. 1999. Neutralizing antibody-independent containment  
 19 of immunodeficiency virus challenges by DNA priming and recombinant pox virus  
 20 booster immunizations. *Nature Medicine.* 5:526-34). To increase the potency of these  
 21 responses, especially the development of high anti-HIV/SIV envelope antibody titers,  
 22 follow-up administration of soluble viral envelope proteins, viral particles or  
 23 recombinant vaccinia-based viruses expressing the HIV/SIV envelope is required  
 24 (Agadjanyan, M. G., N. N. Trivedi, S. Kudchodkar, M. Bennett, W. Levine, A. Lin, J.  
 25 Boyer, D. Levy, K. E. Ugen, J. J. Kim, and D. B. Weiner. 1997. An HIV type 2 DNA  
 26 vaccine induces cross-reactive immune responses against HIV type 2 and SIV. *AIDS*

1 Res. Hum. Retroviruses. 13:1561-1572; Barnett, S. W., J. M. Klinger, B. Doe, C. M.  
2 Walker, L. Hansen, A. M. Duliege, and F. M. Sinangil. 1998. Prime-boost  
3 immunization strategies against HIV. AIDS Res. Hum. Retroviruses. 14 Suppl 3:S299-  
4 309; Letvin, N. L., D. C. Montefiori, Y. Yasutomi, H. C. Perry, M.-E. Davies, C.  
5 Lekutis, M. Alroy, D. L. Freed, C. I. Lord, L. K. Handt, M. A. Liu, and J. W. Shiver.  
6 1997. Potent protective anti-HIV immune responses generated by bimodal HIV  
7 envelope DNA plus protein vaccination. Proc. Natl. Acad. Sci. 94:9378-9383;  
8 Richmond, J. F., S. Lu, J. C. Santoro, J. Weng, S. L. Hu, D. C. Montefiori, and H. L.  
9 Robinson. 1998. Studies of the neutralizing activity and avidity of anti-human  
10 immunodeficiency virus type 1 Env antibody elicited by DNA priming and protein  
11 boosting. J Virol. 72:9092-100; Richmond, J. F. L., F. Mustafa, S. Lu, J. C. Santoro, J.  
12 Weng, M. O'Connell, E. M. Fenyo, J. L. Hurwitz, D. C. Montefiori, and H. L.  
13 Robinson. 1997. Screening of HIV-1 Env glycoproteins for the ability to raise  
14 neutralizing antibody using DNA immunization and recombinant vaccinia virus  
15 boosting. Virology. 230:265-274; Robinson, H. L., D. C. Montefiori, R. P. Johnson, K.  
16 H. Manson, M. L. Kalish, J. D. Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D.  
17 L. Panicali, J. G. Herndon, R. Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand,  
18 and H. M. McClure. 1999. Neutralizing antibody-independent containment of  
19 immunodeficiency virus challenges by DNA priming and recombinant pox virus  
20 booster immunizations. Nature Medicine. 5:526-34). This bimodal method of  
21 immunization elicits responses capable of protecting Rhesus macaques (Rh) from  
22 infection by SHIV ( Letvin, N. L., D. C. Montefiori, Y. Yasutomi, H. C. Perry, M.-E.  
23 Davies, C. Lekutis, M. Alroy, D. L. Freed, C. I. Lord, L. K. Handt, M. A. Liu, and J.  
24 W. Shiver. 1997. Potent protective anti-HIV immune responses generated by bimodal  
25 HIV envelope DNA plus protein vaccination. Proc. Natl. Acad. Sci. 94:9378-9383;  
26 Robinson, H. L., D. C. Montefiori, R. P. Johnson, K. H. Manson, M. L. Kalish, J. D.

1 Lifson, T. A. Rizvi, S. Lu, S. L. Hu, G. P. Mazzara, D. L. Panicali, J. G. Herndon, R.  
2 Glickman, M. A. Candido, S. L. Lydy, M. S. Wyand, and H. M. McClure. 1999.  
3 Neutralizing antibody-independent containment of immunodeficiency virus challenges  
4 by DNA priming and recombinant pox virus booster immunizations. *Nature Medicine*.  
5 5:526-34). However, because during the above method of vaccination both cellular as  
6 well as humoral anti-viral responses were generated, it is unclear whether the recorded  
7 protection was mediated by the cellular and/or humoral anti-viral responses elicited  
8 during DNA immunization. By evaluating and comparing the respective anti-viral  
9 protective roles of these two types of responses, more effective DNA immunization  
10 protocols may be developed.

11

12 Analysis of the crystal structure of the gp120 HIV envelope subunit indicated that  
13 neutralization epitopes are primarily clustered in one face of this protein, which is  
14 naturally occluded within the oligomeric envelope form, i.e., that present on the  
15 surface of virions and infected cells (Kwong, P. D., R. Wyatt, J. Robinson, R. W.  
16 Sweet, J. Sodroski, and W. A. Hendrickson. 1998. Structure of an HIV gp120  
17 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human  
18 antibody. *Nature (London)* 393:648-659; Wyatt, R., P. D. Kwong, E. Desjardins, R.  
19 W. Sweet, J. Robinson, W. A. Hendrickson, and J. G. Sodroski. 1998. The antigenic  
20 structure of the HIV gp120 envelope glycoprotein. *Nature (London)* 393:705-711).  
21 These structural observations are supported by numerous immunochemical and  
22 virological studies (Bou-Habib, D. C., G. Roderiquez, T. Oravesz, P. W. Berman, P.  
23 Lusso, and M. A. Norcross. 1994. Cryptic nature of envelope V3 region epitopes  
24 protects primary monocytotropic human immunodeficiency virus type 1 from antibody  
25 neutralization. *J. Virol.* 68:6006-6013; Moore, J. P., J. A. McKeating, Y. Huang, A.  
26 Askenazi, and D. D. Ho. 1992. Virions of primary human immunodeficiency virus

type 1 isolates resistant to soluble CD4 (sCD4) neutralization differ in sCD4 binding and glycoprotein gp120 retention from sCD4-sensitive isolates. *J. Virol.* 66:235-243; Reitter, J. N., R. E. Means, and R. C. Desrosiers. 1998. A role for carbohydrates in immune evasion in AIDS. *Nat. Med.* 4:679-684; Sattentau, Q. J., and J. P. Moore. 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. *J. Exp. Med.* 174:407-415; Sattentau, Q. J., J. P. Moore, F. Vignaux, F. Traincard, and P. Poignard. 1993. Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding. *J. Virol.* 67:7383-7393; Stamatatos, L., and C. Cheng-Mayer. 1995. Structural modulations of the envelope gp120 glycoprotein of human immunodeficiency virus type 1 upon oligomerization and differential V3 loop epitope exposure of isolates displaying distinct tropism upon virion-soluble receptor binding. *J. Virol.* 69:6191-6198; Sullivan, N., Y. Sun, J. Li, W. Hofmann, and J. Sodroski. 1995. Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates. *J. Virol.* 69:4413-4422; Wyatt, R., J. Moore, M. Accola, E. Desjardin, J. Robinson, and J. Sodroski. 1995. Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. *J. Virol.* 69:5723-5733; Wyatt, R., N. Sullivan, M. Thali, H. Repke, D. Ho, J. Robinson, M. Posner, and J. Sodroski. 1993. Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. *J. Virol.* 67:4557- 4565).

It is towards the enhancement of effective vaccination against HIV-1 that the present invention is directed.



1

2 In another broad aspect of the invention, a vaccine pharmaceutical composition is

3 provided for immunizing an animal against HIV-1 virus, the vaccine pharmaceutical

4 composition comprising an effective heterologous immune-response-eliciting amount

5 of at least one modified HIV-1 envelope protein or fragment thereof, DNA or virus

6 encoding the at least one modified HIV-1 envelope protein or fragment thereof, or a

7 combination thereof, the modified envelope protein or fragment thereof having an

8 HIV-1 envelope protein V2 region deletion; and a pharmaceutically-acceptable carrier

9 or excipient. The modified HIV-1 envelope protein or fragment thereof may be

10 expressed in a mammalian cell. It may be glycosylated. In one embodiment, the

11 modified HIV-1 envelope protein or fragment thereof is from a clade-B HIV-1 strain.

12 In a preferred embodiment, the HIV-1 strain is SF162. By way of non-limiting

13 examples, the modified HIV-1 envelope protein or fragment thereof is SEQ ID No:2

14 or SEQ ID No:4; and a DNA encoding said at least one modified HIV-1 envelope

15 protein or fragment thereof is SEQ ID No:1 or SEQ ID No:3. Immunization or

16 vaccination of an animal with the foregoing vaccine pharmaceutical composition

17 elicits a heterologous immune response to HIV-1. The response comprises a humoral

18 response. In one embodiment, the humoral response comprises neutralizing

19 antibodies. In a preferred embodiment, the elicited antibodies are protective.

20

21 The invention is also directed to a method for assessing whether a compound is

22 capable of generating at least neutralizing antibodies in an animal against at least one

23 heterologous strain of HIV-1 comprising the steps of immunizing the animal with the

24 compound, depleting the animal of its CD8+ cells, and screening the animal for the

25 presence of neutralizing antibodies, or preferably protecting antibodies, to at least one

26 heterologous strain of HIV-1. In one embodiment, the depleting is carried out by





1 schedule: ○: pre-bleeds; ▲: 1 month post the third DNA immunization; ■: 2 weeks  
2 following the first 'boost'; and ♦: 2 weeks following the second 'boost'.

3

4 **Figure 3** shows the depletion of CD8+ T lymphocytes: CD8+ T lymphocytes were  
5 depleted from the vaccinated animals by bolus injection of the anti-CD8 MAb OKT8F  
6 (arrows). The numbers of circulating CD4+ (filled symbols), CD8+ T (open symbols)  
7 and total CD3+ T lymphocytes (asterisks) from vaccinated and unvaccinated animals  
8 was determined in samples collected at various points prior to and following  
9 SHIV162P4-challenge (dashed line).

10

11 **Figure 4 A-B** depicts the viral load and generation anti-HIV envelope antibody titers  
12 following SHIV162P4-exposure: (A) The viral load is expressed as RNA copies per  
13 ml of plasma. Dashed lines indicate the detection limit of this assay (<500 copies per  
14 ml). † The unvaccinated animal AT54 was euthanized 111 days post-challenge  
15 following the development of simian AIDS (SAIDS). The arrow indicates the time at  
16 which CD8+ cells re-appeared in the periphery of the vaccinated animals. (B) The  
17 generation of anti-HIV envelope antibodies following SHIV162P4-challenge was  
18 monitored by SF162ΔV2 gp140-based ELISA methodology. The end-point ELISA  
19 titers are presented.

20

21 **Figure 5** shows the seroconversion of the animals to SIV-gag/pol and HIV env  
22 antigens in the vaccinated and unvaccinated macaques.

23

24 **Figure 6** depicts the development of antibodies in rabbits: The generation of anti-  
25 envelope antibodies was determined by ELISA methodology. Six animals (A1-A6)  
26 were immunized with DNA expressing the unmodified SF162gp140 immunogen and

1 six (A7-A12) with DNA expressing the modified  $\Delta V2gp140$  immunogen. Titers were  
2 determined 2 weeks following each immunization, by ELISA methodology using the  
3 oligomeric SF162gp140 and  $\Delta V2gp140$  proteins. Dashed lines indicate the time of  
4 each immunization.

5  
6 **Figure 7 A-B** depicts neutralization of the SF162 $\Delta V2$  and SF162 viruses by rabbit  
7 sera: Results from neutralization experiments using sera collected following the third  
8 and fifth immunizations against the SF162 $\Delta V2$  (A) and SF162 (B) viruses, are  
9 presented. Data are representative of at least three independent experiments. The  
10 symbols indicate the mean percent neutralization and the standard deviation from  
11 triplicate wells. Dashed lines indicate the 50%, 70% and 90% inhibition of infection.  
12 Dashed lines and asterisks (controls) are neutralization curves obtained with sera  
13 collected from animals that were immunized with the DNA vector alone and are  
14 indicative of non-specific neutralization.

15  
16 **Figure 8** shows the generation of antibodies in Rhesus macaques: The generation of  
17 anti-envelope antibodies in animals (J408 and H445) immunized with the modified  
18  $\Delta V2gp140$  immunogen and two animals (P655 and N472) immunized with the  
19 unmodified SF162gp140 immunogen, as well as control animals (M844 and H473)  
20 immunized with the DNA vector alone, were determined by ELISA methodology  
21 using the corresponding protein. Dashed lines indicate the time of immunizations.  
22 DNA: The animals received three monthly immunizations with DNA vectors  
23 expressing the gp140 form of each immunogen. Control animals received the DNA  
24 vector alone. DNA plus protein: The animals received a fourth DNA immunization  
25 and at the same time they were immunized with the corresponding CHO-produced

1 oligomeric gp140 proteins, adjuvanted in MF-59C. Control animals received adjuvant  
2 alone.

3  
4 **Figure 9 A-B** shows the neutralizing activity of Rhesus macaque sera: The  
5 neutralization activity against the SF162 and SF162ΔV2 viruses of sera collected from  
6 animals immunized with the modified ΔV2gp140 (A) and the unmodified (B)  
7 SF162gp140 immunogens were determined as described in Example 2. Dashed lines  
8 indicate the 50%, 70% and 90% inhibition of infection. Results are representative of  
9 three to five independent experiments. Data indicate the mean and standard deviation  
10 from triplicate wells. Pre-bleeds: sera collected prior to the initiation of vaccination;  
11 second DNA and third DNA: sera collected one month following the second and the  
12 third DNA administration, respectively; 2 and 4 weeks post boost: sera collected 2 and  
13 4 weeks following the DNA plus protein 'booster' immunization, respectively.

14  
15 **Figure 10** depicts the neutralization of heterologous clade B primary HIV-1 isolates  
16 by macaque sera: The neutralization activities of sera collected 2 and 4 weeks  
17 following the DNA plus protein 'booster' immunization, against heterologous to the  
18 vaccine primary HIV-1 isolates, was determined as described in Example 2. Dashed  
19 lines indicate 50%, 70% and 90% inhibition of infection. The values represent the  
20 specific neutralization, which is defined as the difference between the percent virus  
21 neutralization recorded with sera collected following vaccination and that recorded  
22 with sera collected prior to the initiation of vaccination. Data points indicate the mean  
23 percent specific neutralization from two independent experiments.

24  
25 **Figure 11 A-B** shows the generation of binding and neutralizing antibodies following  
26 the second 'booster' immunization with the modified ΔV2gp140 protein: (A) The

1 generation of anti-envelope antibodies in two rhesus macaques (J408 and H445)  
 2 vaccinated with the modified  $\Delta V2gp140$  immunogen were determined by ELISA  
 3 methodology, as described in Example 2. Dashed lines indicate the time of  
 4 immunizations. DNA: The animals received three monthly immunizations with DNA  
 5 vectors expressing the gp140 form of this immunogen; DNA plus protein: the animals  
 6 received a fourth DNA immunization and purified oligomeric  $\Delta V2gp140$  protein; and  
 7 Protein: the animals were immunized with the purified oligomeric  $\Delta V2gp140$  protein  
 8 alone. (B) Neutralization activities against the SF162 $\Delta V2$  and SF162 isolates of sera  
 9 following the second 'boost' were compared to that of sera collected following the  
 10 first 'boost' (see also Figure 4). Non-specific neutralization recorded with pre-  
 11 immunization sera (pre-bleeds) is also shown.

12  
 13 **Figure 12 A-B** shows the presence of anti-V3 loop antibodies in sera collected from  
 14 macaques immunized with the modified  $\Delta V2gp140$  immunogen: The development of  
 15 anti-V3 loop antibodies was determined with the use of an ELISA methodology using  
 16 the V3 loop peptide derived from the SF162/SF162 $\Delta V2$  envelope. (A) First, it was  
 17 examined whether the captured V3 loop peptide interacts with specific anti-V3 loop  
 18 MAbs recognizing linear (447D) and conformational (391-95D) V3 loop epitopes. (B)  
 19 Next, the titer was determined of anti-V3 loop antibodies present in sera collected 2  
 20 and 4 weeks following the first and second boosts from the two vaccinated animals. As  
 21 a comparison the titers of total anti-envelope antibodies present in the same sera were  
 22 also included.

23  
 24 **Figure 13 A-B** shows neutralization of HIV-1 of clades A, E and D by sera from two  
 25 animals immunized with a HIV-1 clade B immunogen-derived modified envelope  
 26 protein having a V2 region deletion.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26

**Figure 14** depicts the polynucleotide sequence of a full-length SF162ΔV2 gp140 envelope protein (SEQ ID No:1).

**Figure 15** depicts the polynucleotide sequence of a SF162ΔV2 gp140 envelope protein fragment (SEQ ID No:3).

**Figure 16** depicts the amino acid sequence of a full-length SF162ΔV2 gp140 envelope protein (SEQ ID No:2).

**Figure 17** depicts the amino acid sequence of a SF162ΔV2 gp140 envelope protein fragment (SEQ ID No:4).

**DETAILED DESCRIPTION OF THE INVENTION**

The inventor herein has made the surprising discovery that animal immunization using modified HIV-1 envelope proteins having a deletion in the V2 (second hypervariable) region elicits potent neutralizing antibodies as part of an anti-HIV-1 envelope-specific immune response. Moreover, the immune response is directed not only to the wild-type form of the immunogen envelope protein, but to other HIV-1 viruses both within and outside of the clade from which the immunogen was derived. This potent, heterologous immune response and in particular the robust humoral response offers a new means for vaccination, among other immunotherapies, for the prophylaxis and treatment of HIV infection. The invention is directed to both DNA, viral and protein vaccines comprising one or more HIV-1 envelope proteins or fragments thereof having a deletion in the V2 region, and to methods for their use.



1 combination of materials in addition to at least one of the immunogens described  
2 herein, and any immunization protocol employing as immunogen a protein or DNA  
3 encoding an HIV-1 viral envelope protein comprising a deletion in the V2 (second  
4 hypervariable) loop (also referred to herein interchangeably as the V2 domain or V2  
5 region). The wild-type sequence of HIV-1 envelope protein candidates for a deletion  
6 in the V2 region in the protein, DNA or virus immunogen as described herein may be  
7 found at <http://idiotype.lanl.gov/>, and all such sequences are incorporated herein by  
8 reference in their entireties as starting sequences for the preparation of an immunogen.  
9 One or a combination of such immunogens may be used together. Furthermore,  
10 various further modifications of the modified (i.e., V2 loop deletion-containing)  
11 envelope proteins of the invention or DNA encoding the modified envelope proteins of  
12 the invention may be made without departing from the invention. For example, the  
13 DNA or viral nucleotide sequence encoding the native envelope leader peptide of the  
14 modified protein can be replaced with a signal peptide of, for example, the human  
15 tissue-specific plasminogen activator gene, for higher protein expression in the  
16 mammalian cells. Other signal peptides may be used. In another embodiment, a  
17 portion of the modified protein or its encoding DNA sequence may be truncated to  
18 provide an immunogen for producing a neutralizing humoral response, and such  
19 modifications are fully embraced herein. Preferably, a fragment is a truncation at the  
20 N-terminal end of the modified protein or DNA or virus encoding the modified  
21 protein, the truncation being from one up to about 30 amino acids, but it not so  
22 limiting, and other truncations are embraced which provide an immunogen with the  
23 immunological properties herein described. Moreover, expression of the DNA  
24 constructs in a mammalian cell, as shown in the examples herein, provides a  
25 glycosylated protein, glycosylated at the asparagine residues indicated in Figures 16  
26 and 17, and the protein immunogen compositions embraced herein include the

1 glycosylated forms of the protein. Thus, the foregoing non-limiting examples of  
 2 variations in the protein and DNA immunogens of the invention which commonly  
 3 comprise a deletion in the V2 loop domain are encompassed by the phrase modified  
 4 protein or fragments thereof, or DNA or virus encoding the modified protein or  
 5 fragments thereof.

6  
 7 The V2 domain is one of the five hypervariable regions of the gp120 subunit of the  
 8 HIV envelope. Its length (number of amino acids) and extent of glycosylation vary  
 9 among HIV isolates. In the case of the SF162 virus, the V2 loop comprises 40 amino  
 10 acids. In the studies herein, 30 amino acids were eliminated from the central region of  
 11 the V2 loop, replacing them by the GAG tripeptide. One of skill in the art may make  
 12 other deletions in the V2 domain of this strain, or deletions in the V2 region in other  
 13 strains, which exhibit the same immune-response-eliciting properties and may readily  
 14 be evaluated for such properties, without deviating from the scope and spirit of the  
 15 invention. As used herein, the abbreviation "ΔV2" refers to a partial or full deletion in  
 16 the V2 domain. A detailed description of the V2 domain of HIV-1 may be found in  
 17 Stamatatos, L., M. Wiskerchen, and C. Cheng-Mayer. 1998. Effect of major deletions  
 18 in the V1 and V2 loops of a macrophage-tropic HIV-1 isolate on viral envelope  
 19 structure, cell-entry and replication. AIDS Res. Hum. Retroviruses 14:1129-1139,  
 20 which is incorporated herein by reference in its entirety.

21  
 22 One non-limiting means by which a modified protein or DNA encoding a modified  
 23 protein comprising the HIV-1 envelope protein may be prepared with a deletion in the  
 24 V2 region may be carried is that described in the aforementioned article or in  
 25 Stamatatos, L., and C. Cheng-Mayer. 1998. An envelope modification that renders a  
 26 primary, neutralization resistant, clade B HIV-1 isolate highly susceptible to



1 neutralization by sera from other clades. J. Virol. 72:7840-7845. By way of non-  
2 limiting example, a modified V2 deletion of the envelope protein of HIV-1 SF162 (a  
3 clade B HIV-1) may be prepared, having the DNA and protein sequence depicted in  
4 SEQ ID No:1 and SEQ ID No:2, respectively. However, other clade B HIV-1  
5 envelope proteins may be similarly modified and the protein or DNA encoding the  
6 protein used as immunogen. Alternatively, HIV-1 envelope proteins of other HIV-1  
7 clades may be used. A selection of HIV-1 proteins and the amino acid sequences of  
8 their envelope proteins may be found in the literature, such as at the Los Alamos  
9 National Laboratories' HIV sequence database, accessible at <http://idiotype.lanl.gov/>.  
10 The present invention embraces these and other HIV-1 envelope proteins as candidates  
11 for deletions in the V2 region for the preparation of a DNA or protein immunogen for  
12 the purposes herein.  
13  
14 Standard molecular biological methods may be used to prepare the HIV-1 envelope  
15 protein with a deletion in the V2 domain, as well as the encoding DNA including  
16 viruses encoding the protein, and the invention herein is not limited as to the method  
17 by which the immunogen is prepared. As used herein, the term DNA vaccine includes  
18 and embraces a viral vaccine comprising DNA encoding the aforementioned protein.  
19 Such methods are well known in the art. As demonstrated herein, one of skill in the  
20 art can readily determine the ability of a DNA or protein immunogen of the invention  
21 to elicit a heterologous HIV-1 immune response in an animal. In the non-limiting  
22 example of the SF162 clade B HIV-1 viral strain, a 30-amino acid deletion from amino  
23 acids T160 to Y189 was prepared, the deleted sequence replaced with a Gly-Ala-Gly  
24 tripeptide. The replacement of the deleted sequences with the aforementioned  
25 tripeptide, or any short peptide, is not required, but may be done for expedience.  
26

1 An animal in which the heterologous viral immune response may be raised is any  
2 animal susceptible to HIV-1 infection or a related virus. Such animals include but are  
3 not limited to humans, non-human primates, and other mammals. In the instance of  
4 humans, the methods of the invention may be carried out with HIV-1, HIV-2, etc.; in  
5 non-human primates, with SHIV-1.  
6  
7 The invention is also directed to a vaccine pharmaceutical composition is provided for  
8 immunizing an animal against HIV-1 virus, the vaccine pharmaceutical composition  
9 comprising an effective heterologous immune response-eliciting amount of at least one  
10 modified HIV-1 envelope protein or fragment thereof, DNA encoding the at least one  
11 modified HIV-1 envelope protein or fragment thereof, or a combination thereof, the  
12 modified envelope protein having a V2 region deletion; and a pharmaceutically-  
13 acceptable carrier or excipient. As used interchangeably herein, the immunogens may  
14 be the full-length or truncated forms of the modified protein or DNA encoding the  
15 modified protein, provided that the deletion in the V2 region elicits a heterologous  
16 immune response. Various selections of useful immunogens are described above. In  
17 one embodiment, the modified HIV-1 envelope protein or fragment is from a clade-B  
18 HIV-1 strain. In a preferred embodiment, the HIV-1 strain is SF162. By way of non-  
19 limiting examples, the modified HIV-1 envelope protein or fragment is SEQ ID No:2  
20 or SEQ ID No:4; and a DNA encoding the at least one modified HIV-1 envelope  
21 protein or fragment is SEQ ID No:1 or SEQ ID No:3. Glycosylation of the protein or  
22 fragment as expressed in mammalian cells is also provided.  
23  
24 The vaccine pharmaceutical composition may comprise one or more of the foregoing  
25 DNA or protein immunogens, together with one or more pharmaceutically-acceptable  
26 carriers, excipients or diluent, to facilitate administration of the vaccine. Moreover,

1 additional components, such as one or more adjuvants, may be included to enhance the  
2 immune response. The selection of the adjuvant will depend on the animal to be  
3 immunized, particularly in humans in which the selection of appropriate adjuvants is  
4 limited. One of skill in the art may select the appropriate pharmaceutically-acceptable  
5 components to include with the immunogen(s) to achieve the desired effect.

7 It is a further object of the present invention to provide a method for assessing whether  
8 a compound, such as an immunogen, is capable of generating protective antibodies  
9 against heterologous strains of HIV-1. The method is carried out by immunizing an  
10 animal with an immunogen, depleting the animal of its CD8+ T-lymphocytes, and then  
11 screening the animal for the presence at least of protective antibodies, and preferably  
12 the presence of protective antibodies, to at least one heterologous strain of HIV-1. The  
13 depleting may be carried out by administering to the animal anti-CD8 monoclonal  
14 antibodies. The compound may be an HIV-derived polypeptide or fragment thereof,  
15 such as but not limited to a DNA vaccine wherein the DNA vaccine encodes an HIV-  
16 derived polypeptide or fragment thereof. The immunization protocol may comprise a  
17 DNA vaccine, a viral vaccine, a protein, any fragments thereof, any combination  
18 thereof, and a protocol in which either or both are administered sequentially in order to  
19 induce an immune response. In a non-limiting embodiment, the neutralizing  
20 antibodies are protective antibodies. The method in which eliciting of protective  
21 antibodies is evaluated may be carried out in an animal such as a primate or other  
22 animal capable of generating protective antibodies to HIV, but it is not so limiting. As  
23 noted above, the foregoing method may be utilized to assess the effectiveness of a  
24 DNA and/or protein immunogen of the invention.

1 As described in the examples below, the observation that the lowest levels of peak  
 2 plasma viremia were recorded in a animal vaccinated with the  $\Delta$ V2 immunogen and  
 3 whose serum had the strongest neutralizing activity against SHIV162P4 at the day of  
 4 challenge, indicates that neutralizing antibodies played an important protective role  
 5 during the first 7 days post-challenge. The fact that strong anamnestic anti-HIV  
 6 envelope responses were developed immediately following SHIV162P4-challenge  
 7 indicates that antibodies contributed to the rapid viral-clearance to undetectable levels.  
 8 However, because the CD8+ lymphocytes reappeared in the periphery of the  
 9 vaccinated animals 7 days post-challenge, they may also have contributed to this rapid  
 10 viral clearance.

11  
 12 Moreover, the herein studies also show an immune response to HIV-1 of different  
 13 clades than that from which the immunogen was prepared, referred to herein as a  
 14 heterologous immune response.

15  
 16 These studies highlight the important protective role of non-CD8-mediated DNA-  
 17 based vaccine-induced anti-HIV envelope responses and demonstrate the feasibility to  
 18 develop an effective anti-HIV vaccine for human use for the prophylaxis and treatment  
 19 of HIV infection. As noted above, the strategy of using a modified envelope protein  
 20 with a  $\Delta$ V2 loop deletion is a strategy that may be employed for any V2-loop-bearing  
 21 envelope protein, and the present invention embraces any and all such uses, as well as  
 22 pharmaceutical compositions comprising a  $\Delta$ V2 loop deletion modified protein or  
 23 DNA vaccine, or combination, for the purposes of eliciting an immune response.

24  
 25 In the studies described herein, immunogenicity was compared between soluble  
 26 oligomeric gp140 envelope proteins derived from related neutralization-resistant

1 (SF162) and neutralization-susceptible (SF162ΔV2) viruses (Stamatatos, L., and C.  
2 Cheng-Mayer. 1998. An envelope modification that renders a primary, neutralization  
3 resistant, clade B HIV-1 isolate highly susceptible to neutralization by sera from other  
4 clades. J. Virol. 72:7840-7845). The only difference between the two immunogens is  
5 the absence of 30 amino acids from the V2 loop of the SF162ΔV2-derived immunogen  
6 (Stamatatos, L., and C. Cheng-Mayer. 1998. An envelope modification that renders a  
7 primary, neutralization resistant, clade B HIV-1 isolate highly susceptible to  
8 neutralization by sera from other clades. J. Virol. 72:7840-7845). Immunization  
9 studies were first performed in rabbits, where it was observed that although both  
10 proteins elicited similar titers of binding antibodies, the modified immunogen elicited  
11 higher titers of neutralizing antibodies against isolates expressing not only the  
12 modified SF162ΔV2 envelope, but also the unmodified parental SF162 envelope.  
13  
14 In rabbits, both the unmodified SF162gp140 and the modified ΔV2gp140 immunogens  
15 elicited neutralizing antibodies against several heterologous primary HIV-1 isolates,  
16 but the potential of the modified immunogen to do so was greater, and importantly, not  
17 previously described or expected. Thus, not only a greater number of animals  
18 vaccinated with the modified immunogen elicited cross-reactive neutralizing  
19 antibodies, but also the breadth and potency of the cross-neutralizing responses were  
20 higher in sera collected from these animals than animals immunized with the  
21 unmodified immunogen. The modified immunogen more effectively elicits antibodies  
22 recognizing neutralization epitopes that are conserved among several HIV isolates  
23 than the unmodified immunogen.  
24  
25 The vaccination studies conducted in rhesus macaques confirm the observations made  
26 in rabbits, that the modified ΔV2gp140 immunogen is more effective than the



1 expression in mammalian cells. At week 27 the animals were immunized one  
2 additional time with DNA and with the CHO-produced, purified oligomeric  
3 SF162ΔV2 gp140 protein (100 μg) mixed with the MF-59C adjuvant. At week 38 the  
4 animals were immunized one additional time with the adjuvanted protein alone.  
5  
6 The development of binding antibodies was evaluated by ELISA methodologies  
7 (Stamatatos, L., and C. Cheng-Mayer. 1995. Structural modulations of the envelope  
8 gp120 glycoprotein of human immunodeficiency virus type 1 upon oligomerization  
9 and differential V3 loop epitope exposure of isolates displaying distinct tropism upon  
10 virion-soluble receptor binding. *J. Virol.* 69:6191-6198).  
11  
12 Antibodies were detectable following the second DNA immunization and their titers  
13 did not increase following the third DNA immunization (**Figure 1**). During the  
14 following five months the titers decreased gradually, but were always detectable. The  
15 first 'boost' increased the titers by approximately 1-2 log<sub>10</sub> from the peak value  
16 recorded following the third DNA immunization. The titers gradually decreased and  
17 leveled off during the following 11 weeks, at which point the animals received a  
18 second 'boost', which further increased the antibody titers. Neutralizing antibodies  
19 (NA) were evaluated using the 'activated PBMC-target' assay (Stamatatos, L., and C.  
20 Cheng-Mayer. 1998. An envelope modification that renders a primary, neutralization  
21 resistant, clade B HIV-1 isolate highly susceptible to neutralization by sera from other  
22 clades. *J. Virol.* 72:7840-7845), using pre-immunization sera to correct for non-  
23 specific neutralization (**Figure 2**). Following the third DNA-immunization, the NA  
24 titers in animal H445 were lower than those in animal J408, even though the binding  
25 antibody titers were similar between the two animals. The NA titers against both  
26 SF162ΔV2 and SF162 increased significantly during the subsequent 'boosts'.

1 Vaccine-specific proliferative responses were also recorded in both animals.

2 Stimulation indexes (S.I.) of 5 and 10 were recorded following the first 'boost' in

3 animals J408 and H445, respectively. The second 'boost' increased the potency of

4 these responses in animal H445 (S.I. of 25), but not in animal J408 (S.I. of 5).

5

6 To evaluate the protective role of the anti-HIV envelope antibodies elicited by the

7 vaccine of the invention, CD8+ cells were depleted from the vaccinated animals prior

8 to viral-challenge (**Figure 3**). CD8-depletion was achieved by three intravenous

9 administrations of the anti-CD8 MAb OKT8F (2 mg / kg) at daily intervals (Jin, X., D.

10 E. Bauer, S. E. Tuttleton, S. Lewin, A. Gettie, J. Blanchard, C. E. Irwin, J. T. Safrit, J.

11 Mittler, L. Weinberger, L. G. Kostrikis, L. Zhang, A. S. Perelson, and D. D. Ho. 1999.

12 Dramatic rise in plasma viremia after CD8(+) T cell depletion in simian

13 immunodeficiency virus-infected macaques. J Exp Med. 189:991-8). CD8+ T

14 lymphocytes remained undetectable for approximately 10 days. Concomitantly, a

15 decrease was recorded in the total number of circulating CD3+ T cells. This indicates

16 that the recorded depletion of CD8+ T cells from the periphery is due to their actual

17 elimination. Although CD8-depletion from the lymph nodes was not evaluated, it was

18 previously demonstrated that a concomitant depletion of CD8+ T cells from the

19 periphery and lymph nodes occurs when anti-CD8 MAbs are introduced in the blood

20 circulation of macaques (Matano, T., R. Shibata, C. Siemon, M. Connors, H. C. Lane,

21 and M. A. Martin. 1998. Administration of an anti-CD8 monoclonal antibody

22 interferes with the clearance of chimeric simian/human immunodeficiency virus

23 during primary infections of rhesus macaques. J. Virol. 72:164-169; Schmitz, J. E., M.

24 J. Kuroda, S. Santra, V. G. Sasseville, M. A. Simon, M. A. Lifton, P. Racz, K. Tenner-

25 Racz, M. Dalesandro, B. J. Scallan, J. Ghayeb, M. A. Forman, D. C. Montefiori, E. P.



1 Rieber, N. L. Letvin, and K. A. Reimann. 1999. Control of viremia in simian  
2 immunodeficiency virus infection by CD8+ lymphocytes. *Science*. 283:857-60).  
3  
4 One day following the last administration of OKT8F, the immunized and two un-  
5 immunized naive animals were challenged intravenously with 100 TCID<sub>50</sub> of a cell-  
6 free stock of the SHIV162P4 virus (Harouse, J. M., A. Gettie, R. C. Tan, J. Blanchard,  
7 and C. Cheng-Mayer. 1999. Distinct pathogenic sequela in rhesus macaques infected  
8 with CCR5 or CXCR4 utilizing SHIVs. *Science*. 284:816-9). This isolate was  
9 neutralized by 50% and 90% by sera (1:5 dilution) collected at the day of challenge  
10 from animals H445 and J408, respectively.  
11  
12 Both vaccinated and unvaccinated animals became infected; however, differences in  
13 the peak viral load levels and viral set points were noted between the two groups  
14 (**Figure 4A**). Eleven days post-challenge, plasma viremia in the vaccinated animal  
15 H445 was lower by 2 and 4 log<sub>10</sub> as compared to that of the unvaccinated animals  
16 A141 and AT54, respectively, while the vaccinated animal J408 was aviremic. At peak  
17 viremia, viral plasma levels in the vaccinated animals were 1- 4 log<sub>10</sub> lower than in the  
18 unvaccinated animals. Following peak viremia, an initial rapid decrease followed by a  
19 more gradual decrease in plasma viral loads was recorded in the unvaccinated animal  
20 A141, while sustained high viral loads were recorded in the second unvaccinated  
21 animal AT54. A very rapid decrease to undetectable levels was recorded in both  
22 vaccinated animals within 35 days post-challenge.  
23  
24 Concomitant with the appearance of plasma viremia in the vaccinated animal H445, a  
25 rapid increase (by approximately 5 fold) of the anti-HIV envelope antibody titers was  
26 monitored (**Figure 4B**). Subsequently, as the viral load in this animal decreased to



1 to neutralizing antibodies, envelope-specific antibodies without neutralizing activity  
 2 may have been elicited by the vaccine of the invention and may also have contributed  
 3 in viral clearance. The fact that strong anamnestic anti-HIV envelope responses were  
 4 developed in animal H445 immediately following SHIV-challenge indicates that  
 5 antibodies contributed to the rapid viral-clearance to undetectable levels. However,  
 6 because the CD8+ lymphocytes reappeared in the periphery of the vaccinated animals  
 7 7 days post-challenge, they may also have contributed to this rapid viral clearance.

8  
 9 These studies highlight the important protective role of non-CD8-mediated DNA-  
 10 vaccine-induced anti-HIV envelope responses and demonstrate the feasibility to  
 11 develop an effective anti-HIV vaccine.

## 13 EXAMPLE 2

14 In the studies presented here, the immunogenic potential of the unmodified SF162 is  
 15 compared to that of modified SF162 $\Delta$ V2 (from here on designated as  $\Delta$ V2) envelopes.  
 16 Using the gene-gun vaccination methodology rabbits were immunized with the gp140  
 17 form of the SF162 and  $\Delta$ V2 envelopes. Both immunogens elicited the generation of  
 18 similar antibody titers, but the modified immunogen elicited higher titers of  
 19 neutralizing antibodies against the parental SF162 virus than the unmodified  
 20 immunogen. Additionally, the  $\Delta$ V2-derived modified immunogen was more effective  
 21 than the SF162-derived unmodified immunogen in generating antibodies capable of  
 22 neutralizing heterologous primary HIV-1 isolates.

23  
 24 The immunogenicity of these two antigens was also evaluated in Rhesus macaques, an  
 25 animal model more closely related to humans and more suitable for HIV-vaccine  
 26 studies, using the DNA-prime followed by protein-boosting vaccination methodology.



1 vaccines expressing human immunodeficiency virus type 1 envelope glycoprotein with  
2 and without deletions in the V1/V2 and V3 regions. *AIDS Res. Hum. Retroviruses*  
3 14:151-155). The DNA vector used to immunize Rhesus macaques is derived from the  
4 pCMVKm2 vector (Chapman, B. S., R. M. Thayer, K. A. Vincent, and N. L.  
5 Haigwood. 1991. Effect of intron A from human cytomegalovirus (Towne)  
6 immediate-early gene on heterologous expression in mammalian cells. *Nucleic Acids*  
7 *Res.* 19:3979-86; zur Megede, J., M. C. Chen, B. Doe, M. Schaefer, C. E. Greer, M.  
8 Selby, G. R. Otten, and S. W. Barnett. 2000. Increased expression and  
9 immunogenicity of sequence-modified human immunodeficiency virus type 1 gag  
10 gene. *J. Virol.* 74:2628-35). Both DNA plasmids contain the human CMV  
11 enhancer/promoter elements and the native leader peptide of the HIV envelope was  
12 replaced with that derived from the tissue-specific plasminogen activator gene. In the  
13 case of macaque-immunizations, the DNA construct was codon-optimized for high  
14 expression in mammalian cells. Both DNA vectors express the gp140 ectodomain  
15 form of the HIV envelope immunogen, with an intact gp120-gp41 cleavage site.  
16  
17 Protein-boosting immunizations were performed only in rhesus macaques to increase  
18 the titer of antibodies elicited following the DNA-phase of immunization. For this  
19 purpose, the  $\Delta$ V2 gp140 protein was produced in CHO cells and purified as stable  
20 soluble trimers. To increase, however, the stability of these secreted oligomers, the  
21 gp120-gp41 cleavage site was eliminated by mutagenesis (Earl, P. L., S. Koenig, and  
22 B. Moss. 1991. Biological and immunological properties of human immunodeficiency  
23 virus type 1 envelope glycoprotein: analysis of proteins with truncations and deletions  
24 expressed by recombinant vaccinia viruses. *J Virol* 65:31-41; Earl, P. L., and B. Moss.  
25 1993. Mutational analysis of the assembly domain of the HIV-1 envelope  
26 glycoprotein. *AIDS Res. Hum. Retroviruses* 9:589-594; Stamatatos, L., M. Lim, and

1 C. Cheng-Mayer. 2000. Generation and structural analysis of soluble oligomeric  
2 envelope proteins derived from neutralization-resistant and neutralization-susceptible  
3 primary HIV-1 isolates. *AIDS Res. Hum. Retroviruses* 16:981-994).

4

5 Immunizations: a) Rabbits: Using the gene-gun vaccination methodology (Lu, S., R.  
6 Wyatt, J. F. L. Richmond, F. Mustafa, S. Wang, J. Weng, D. C. Montefiori, J.  
7 Sodroski, and H. L. Robinson. 1998. Immunogenicity of DNA vaccines expressing  
8 human immunodeficiency virus type 1 envelope glycoprotein with and without  
9 deletions in the V1/V2 and V3 regions. *AIDS Res. Hum. Retroviruses* 14:151-155) the  
10 animals received 5 DNA immunizations (each immunization consisting of 36 shots of  
11 0.5 µg DNA each) at weeks 0, 4, 8, 18 and 22. Blood was drawn two weeks following  
12 each immunization. Six animals (A1-A6) were immunized with the unmodified  
13 SF162gp140 immunogen and six animals (A7-A12) with the modified ΔV2gp140  
14 immunogen. Two animals (A13 and A14) served as controls and were immunized with  
15 the DNA vector alone.

16

17 b) Rhesus macaques: Animals H445 and J408 were immunized with the modified  
18 ΔV2gp140 immunogen, animals N472 and P655 with the unmodified SF162gp140  
19 immunogen and animals M844 and H473 with the DNA vector alone. Prior to the  
20 initiation of immunizations, the animals were tested for antibodies to various Simian  
21 viruses such as SIV, type D retroviruses and STLV-1. Animals vaccinated with the  
22 modified envelope were immunized with DNA at weeks 0, 4 and 8, and animals  
23 vaccinated with the unmodified envelope were immunized with DNA at weeks 0, 4  
24 and 9. The DNA (2 mg DNA in 1ml of endotoxin-free water each time per animal)  
25 was administered both intradermally (i.d.) at two sites (2 x 0.2 mg) and  
26 intramuscularly (i.m.) (2 x 0.8 mg in the quadriceps muscles). Animals were

1 immunized a fourth time with DNA and at the same time with the purified oligomeric  
2  $\Delta$ V2gp140 or SF162gp140 proteins mixed with the MF-59C adjuvant. The proteins  
3 (0.1 mg of purified protein in 0.5 ml total volume per animal) were administered i.m.  
4 in the deltoids. The control animals received only adjuvant. This DNA plus protein  
5 'booster' immunization took place at week 27 for animals vaccinated with the  
6 modified immunogen and at week 48 for animals immunized with the unmodified  
7 immunogen. At week 38 the animals immunized with the modified, but not those  
8 immunized with the unmodified, immunogen were immunized one additional time  
9 with the adjuvanted protein alone (no DNA).

10

11 Antibody determination: a) Anti-gp140 antibodies: Titers were determined throughout  
12 the immunization protocol using an ELISA methodology as previously described  
13 (Stamatatos, L., and C. Cheng-Mayer. 1995. Structural modulations of the envelope  
14 gp120 glycoprotein of human immunodeficiency virus type 1 upon oligomerization  
15 and differential V3 loop epitope exposure of isolates displaying distinct tropism upon  
16 virion-soluble receptor binding. J. Virol. 69:6191-6198; Stamatatos, L., M.  
17 Wiskerchen, and C. Cheng-Mayer. 1998. Effect of major deletions in the V1 and V2  
18 loops of a macrophage-tropic HIV-1 isolate on viral envelope structure, cell-entry and  
19 replication. AIDS Res. Hum. Retroviruses 14:1129-1139). Briefly, purified soluble  
20 oligomeric  $\Delta$ V2gp140 and SF162gp140 proteins were used to coat ELISA plates  
21 (Immulon 2HB) (0.2  $\mu$ g of protein in 0.1 ml of 100 mM NaHCO<sub>3</sub>, pH 8.5) by an  
22 overnight incubation at 4°C. Non-adsorbed protein molecules were removed by  
23 washing with TBS and the wells were blocked with SuperBlock (SB) (Pierce). Heat-  
24 inactivated (56°C for 35 minutes) sera collected from the immunized animals were  
25 serially diluted in SB and added to the wells (0.1 ml per well) for one hour at 37°C. In  
26 the case of rabbits, sera from control animals receiving the DNA vector alone were

1 used as negative controls. In the case of macaques, pre-immunization sera were used  
 2 as negative controls. Unbound antibodies were removed by TBS-washing and the  
 3 envelope-bound antibodies were detected with the use of goat anti-human (in the case  
 4 of Rhesus sera) or anti-rabbit (in the case of rabbit sera) IgG coupled to alkaline  
 5 phosphatase antibodies (Zymed Immunochemicals) as previously described  
 6 (Stamatatos, L., and C. Cheng-Mayer. 1995. Structural modulations of the envelope  
 7 gp120 glycoprotein of human immunodeficiency virus type 1 upon oligomerization  
 8 and differential V3 loop epitope exposure of isolates displaying distinct tropism upon  
 9 virion-soluble receptor binding. *J. Virol.* 69:6191-6198). The OD490nm of each well  
 10 was recorded with a Bioluminometer (Molecular Dynamics). A plot of the OD490nm  
 11 signals versus serum-dilution was generated and end-point antibody titers were  
 12 determined as the highest post-immunization serum dilution that produces an  
 13 OD490nm value three times that of the OD 490nm produced by the pre-immunization  
 14 sera at their lowest dilution. Sera from various stages of immunization were tested at  
 15 the same time.

16  
 17 Neutralization assays: Neutralization assays were performed using as target cells  
 18 human PBMC activated for three days with PHA (Sigma, 3 µg/ml) as previously  
 19 described (Mascola, J. R., M. G. Lewis, G. Stiegler, D. Harris, T. C. VanCott, D.  
 20 Hayes, M. K. Louder, C. R. Brown, C. V. Sapan, S. S. Frankel, Y. Lu, M. L. Robb, H.  
 21 Katinger, and D. L. Birx. 1999. Protection of Macaques against pathogenic  
 22 simian/human immunodeficiency virus 89.6PD by passive transfer of neutralizing  
 23 antibodies. *J. Virol.* 73:4009-18; Mascola, J. R., S. W. Snyder, O. S. Weislow, S. M.  
 24 Belay, R. B. Belshe, D. H. Schwartz, M. L. Clements, R. Dolin, B. S. Graham, G. J.  
 25 Gorse, M. C. Keefer, M. J. McElrath, M. C. Walker, K. F. Wagner, J. G. McNeil, F. E.  
 26 McCutchan, D. S. Burke and the NIAID AIDS vaccine evaluation group. 1996.







1  
2 Results: Generation of antibodies in rabbits: Both the SF162- and  $\Delta$ V2-derived  
3 immunogens elicited high titers of antibodies capable of binding to both the  
4 oligomeric  $\Delta$ V2gp140 and SF162gp140 proteins (**Figure 6**). As expected, variations in  
5 the antibody-titers were recorded throughout the vaccination schedule in animals  
6 belonging to either group. However, no statistically significant differences in antibody  
7 titers were recorded between the two animal groups throughout the immunization  
8 schedule. The antibody titers in each animal, regardless of whether it was immunized  
9 with the modified or the unmodified immunogen, were very weak during the first two  
10 immunizations (at 0 and 4 weeks). The fourth immunization (at 18 weeks) resulted in  
11 an increase in antibody titers, as compared to the third immunization (8 weeks),  
12 between 2 and 3  $\log_{10}$  in both animal groups. The fifth immunization (22 weeks)  
13 increased the antibody titers, as compared to the fourth immunization, against the  
14 SF162gp140 antigen (by less than 1  $\log_{10}$ ), but not against the  $\Delta$ V2gp140 protein. At  
15 the end of the vaccination schedule, very potent end-point ELISA binding antibody  
16 titers in the order of  $10^5$ - $10^6$  were recorded in both animal groups against both  
17 antigens. Thus, it appears that in rabbits, based on the assay used here to determine  
18 antibody titers, the modified immunogen is as effective as the unmodified immunogen  
19 in eliciting the generation of antibodies even though the former immunogen lacks 30  
20 amino acids from the V2 loop.

21  
22 Neutralizing activity in rabbit sera against the SF162 and SF162 $\Delta$ V2 isolates: Both  
23 immunogens generated neutralizing antibodies against the SF162 $\Delta$ V2 virus following  
24 the third DNA-immunization (**Figure 7A**). A trend towards higher neutralization titers  
25 in the modified immunogen-vaccinated group was recorded. Thus, the mean serum  
26 dilution at which 70% inhibition of infection was recorded (and standard error) for

1 SF162gp140- and  $\Delta$ V2gp140-immunized animals was 179 (+/- 34) and 483 (+/- 148),  
2 respectively. At this stage of vaccination, while 2 (A8 and A9) out of 6 animals  
3 immunized with the modified immunogen elicited neutralizing antibodies against the  
4 parental SF162 isolate, none of the animals immunized with the unmodified  
5 immunogen elicited antibodies capable of doing so (**Figure 7B**). However, the number  
6 of animals that generated neutralizing antibodies against the SF162 and SF162 $\Delta$ V2  
7 viruses increased with each subsequent immunization, so that at the end of the  
8 immunization schedule (i.e., after the fifth immunization) all animals had generated  
9 neutralizing antibodies against the SF162 virus. In addition, the neutralization potency  
10 of each serum, regardless of whether the animal was vaccinated with the modified or  
11 unmodified immunogen, increased with each immunization.

12  
13 At the end of the immunization schedule, sera collected from rabbits immunized with  
14 the modified immunogen had higher neutralization potency against the SF162 $\Delta$ V2 as  
15 well as against SF162 viruses, than the sera collected from animals immunized with  
16 the unmodified immunogen. Six out of six animals immunized with the modified  
17 immunogen elicited antibodies capable of neutralizing the SF162 $\Delta$ V2 virus between  
18 70% and 100% at a 1:5,000 dilution (**Figure 7A**). In contrast, at the same serum  
19 dilution only one (A1) of the six animals vaccinated with the unmodified envelope  
20 developed antibody responses able to neutralize SF162 $\Delta$ V2 infection, and that by only  
21 50%. The remaining five animals in this group failed to elicit antibody responses  
22 potent enough to neutralize SF162 $\Delta$ V2-infection to any significant extent at this  
23 dilution. Differences in neutralizing potential between sera collected from animals  
24 immunized with the modified immunogen and those immunized with the unmodified  
25 immunogen were also evident when their ability to neutralize the SF162 virus was  
26 compared (**Figure 7B**). Sera collected from four (A8, A9, A10 and A12) out of six

1 animals immunized with the modified antigen neutralized SF162-infection between  
2 70% and 90% at 1:100 to 1:300 dilutions. In contrast, none of the sera collected from  
3 animals immunized with the unmodified antigen could inhibit SF162-infection by  
4 70%-90% at the same dilutions.

5 Generation of cross-reactive neutralizing antibodies in rabbits: The fact that the  
6 SF162 $\Delta$ V2-derived envelope immunogen was capable of eliciting higher titers of  
7 neutralizing antibodies against the parental SF162 isolate (which expresses the full  
8 envelope) than the immunogen derived from the SF162 isolate itself, prompted us to  
9 examine whether the modified immunogen was also more effective in eliciting cross-  
10 reactive neutralizing antibodies, i.e., antibodies capable of neutralizing heterologous to  
11 the vaccine primary HIV-1 isolates. Several such isolates were tested whose  
12 neutralization susceptibility to various monoclonal antibodies was previously  
13 documented (D'Souza, M. P., D. Livnat, J. A. Bradac, and S. H. Bridges. 1997.

14 Evaluation of monoclonal antibodies to human immunodeficiency virus type 1  
15 primary isolates by neutralization assays: performance criteria for selecting candidate  
16 antibodies for clinical trials. AIDS Clinical Trials Group Antibody Selection Working  
17 Group. J. Infect. Dis. 175:1056-62). Only two (92US714 and the 92HT593) out of the  
18 six isolates, examined where neutralized by antibodies elicited by the unmodified  
19 immunogen (Table 1, below).

Table 1. Generation of cross-reactive neutralizing antibodies in rabbits								
		ISOLATES						
	Animals	91US054 (50) (80)	92US657 (50) (80)	92US660 (50) (80)	92HT593 (50) (80)	91US056 (50) (80)	92US714 (50) (80)	
Unmodified SF162gp140	A1	-	-	-	-	-	-	-
	A2	-	-	-	+	-	+	-
	A3	-	-	-	-	-	+	-
	A4	-	-	-	-	-	+	-
	A5	-	-	-	+	+	+	+
	A6	-	-	-	-	-	+	-
Modified ΔV2gp140	A7	+	+	-	+	+	+	-
	A8	+	+	-	+	+	+	+
	A9	+	-	+	+	+	+	+
	A10	-	+	-	+	-	+	+
	A11	-	-	-	-	-	-	-
	A12	-	-	-	-	-	-	-

1

2 The neutralizing activity was evaluated at 1:10 dilution, taking into consideration the  
3 non-specific neutralization recorded with sera collected from animals vaccinated with  
4 the DNA vector alone (see Materials and Methods for details). (-): 50% specific  
5 neutralization was not recorded. (+): 50% or 80% specific neutralization was  
6 recorded. Results are from three independent neutralization experiments.

7

8 With the exception of animal A1, all other animals developed neutralizing antibodies  
9 against 92US714, while only animals A2 and A5 generated neutralizing antibodies  
10 against 92HT593. In contrast, four out of the six animals immunized with the modified  
11 ΔV2gp120 immunogen generated cross-reactive neutralizing antibodies against most  
12 of the heterologous isolates tested. In addition, the neutralization potency of sera

collected from animals immunized with the modified immunogen was higher than that of sera collected from animals immunized with the unmodified immunogen (see Table 1, above). Thus, although 80% inhibition of infection was frequently recorded with the former sera, this level of inhibition was recorded in only two instances (sera from animal A5 versus the 92US714 and 92HT593 isolates).

7 Development of antibodies in Rhesus macaques vaccinated with the modified  
8  $\Delta$ V2gp140 immunogen: The above results prompted an evaluation of the  
9 immunogenic potential of the unmodified SF162gp140 and modified  $\Delta$ V2gp140  
10 antigens in Rhesus macaques, an animal model where the protective potential of  
11 vaccine-elicited antibodies can eventually be evaluated. Macaques were vaccinated  
12 with these two immunogens using the DNA-prime followed by protein-boosting  
13 vaccination methodology.

Envelope-specific antibodies became detectable following the second DNA immunization (**Figure 8**). At this stage, end point ELISA titers in animals immunized with the modified antigen (animals J408 and H445) were in the order of 1:2,000. In contrast, in animals immunized with the unmodified envelope (animals N472 and P655), antibodies were only detectable in animal N472 (end point ELISA titers in the order of 1:500). With the exception of animal H445, the third DNA immunization did not further increase the antibody titers. Anti-gp120 and anti-gp41 antibodies were generated synchronously during DNA immunization.

24 During the subsequent five to ten months of observation, antibodies were undetectable  
25 in animals immunized with the unmodified SF162gp140 immunogen, while in animals

1 immunized with the modified  $\Delta$ V2gp140 immunogen the antibodies were always  
2 detectable, but their titers declined over time.  
3  
4 Following the DNA plus protein 'booster' immunization, the antibody titers increased  
5 significantly in all animals. At their peak value (reached within 2-4 weeks post-  
6 'boosting'), end-point ELISA antibody titers in animals immunized with the modified  
7  $\Delta$ V2gp140 immunogen were 1:30,000 for animal J408 and 1:110,000 for animal  
8 H445. The titers decreased gradually over time and remained stable at approximately  
9 1:8,000 for several weeks in both animals. Higher peak antibody titers were recorded  
10 in animals vaccinated with the unmodified SF162gp140 immunogen (end-point  
11 ELISA antibody titers of 1:150,000 in animal N472 and 175,000 in animal P655).  
12 During the following 7 weeks of observation the antibody titers decreased more  
13 rapidly in both animals to approximately 1:35,000. Thus, in contrast to what was  
14 recorded in rabbits, in macaques the unmodified immunogen generated higher titers of  
15 binding antibodies than the modified immunogen.  
16  
17 As expected anti-HIV envelope antibodies were not generated in control animals  
18 (M844 and H473) immunized with the DNA vector alone.  
19  
20 Neutralizing activity of macaque sera against the homologous SF162 $\Delta$ V2 and parental  
21 SF162 isolates: During the DNA phase of immunization, only animals immunized  
22 with the modified  $\Delta$ V2gp140 immunogen elicited neutralizing antibodies against the  
23 SF162 and SF162 $\Delta$ V2 viruses (**Figure 9A-B**). Following the second DNA  
24 immunization, animal J408 developed neutralizing antibodies against the homologous  
25 SF162 $\Delta$ V2, but not the parental SF162, isolate (**Figure 9A**). The titer of neutralizing  
26 antibodies in animal J408 increased following the third DNA immunization, at which



1 point neutralization of both isolates was recorded, although the titers of binding  
2 antibodies did not increase in parallel (**Figure 9B**). In contrast, much weaker  
3 neutralizing antibody responses against the SF162 $\Delta$ V2 and no neutralizing responses  
4 against the SF162 virus were elicited in animal H445, even though this animal  
5 generated similar titers of binding antibodies to those generated in animal J408  
6 (**Figure 9B**).

7  
8 Two weeks following the DNA plus protein 'booster' immunization sera collected  
9 from animals immunized with either immunogen inhibited SF162 $\Delta$ V2-infection. The  
10 neutralization potency of sera collected from animals immunized with the modified  
11 immunogen was higher than that of sera collected from animals immunized with the  
12 unmodified immunogen. For example, 50% inhibition of SF162 $\Delta$ V2-infection was  
13 recorded at dilutions of 1:2,000 to 1:5,000 from the former sera, but this level of  
14 inhibition was not recorded at this dilutions with sera collected from the latter sera.  
15 Both  $\Delta$ V2gp140-immunized animals generated strong neutralizing antibodies against  
16 the parental SF162 virus, while only one (N472) of the two animals immunized with  
17 the SF162gp140 immunogen generated neutralizing antibodies against this virus.  
18 Changes in the neutralizing potency of these sera were not recorded during the  
19 subsequent two weeks, even tough changes in the antibody titer levels were detectable  
20 during this period (**Figure 9**). Control animals (M844 and H473) vaccinated with the  
21 vector alone did not develop neutralizing antibodies.

22  
23 Neutralization of heterologous primary HIV-1 isolates by macaques sera: The breath  
24 of the neutralizing antibody responses elicited in macaques immunized with the  
25 modified and unmodified immunogens, was evaluated by comparing the ability of sera  
26 collected from macaques immunized with these two immunogens to block infection of

heterologous primary clade B HIV-1 isolates. The susceptibility of these isolates to neutralization by various MAbs was previously reported (D'Souza, M. P., D. Livnat, J. A. Bradac, and S. H. Bridges. 1997. Evaluation of monoclonal antibodies to human immunodeficiency virus type 1 primary isolates by neutralization assays: performance criteria for selecting candidate antibodies for clinical trials. AIDS Clinical Trials Group Antibody Selection Working Group. J. Infect. Dis. 175:1056-62). During the serum neutralization experiments, in parallel the susceptibility was evaluated of these isolates to neutralization by two of the most commonly used primary-isolate neutralizing MAbs (2F5 and 2G12) (Table 2).

10

11

Table 2. Neutralization of heterologous primary HIV-1 isolates by macaque sera										
			IMMUNOGEN							
	Mab		ΔV2GP140				SF162GP140		SF2gp120	
ISOLATE S	2F5 2G12		J408 (A) <sup>&amp;</sup>	(B)	H445 (A)	(B)	P655 (A)	N472 (A)	L714	L814
91US056 (R5)	60	70	90	-	65	-	-	-	-	-
92US714 (R5)	70	20	85	-	85	-	-	-	-	-
92US660 (R5)	75	70	50	-	80	-	-	-	-	-
92HT593 (R5X4)	75	80	-	-	-	-	-	-	-	-
92US657 (R5)	NT	NT	-	-	-	65	-	-	-	-
BZ167 (R5X4)	90	75	NT	-	NT	80	NT	NT	NT	NT
ADA (R5)	NT	NT	90	50	90	80	NT	NT	NT	NT

12

Values represent the percent neutralization of a given HIV-1 isolate by sera (1:10 dilution) collected from animals immunized with the modified  $\Delta$ V2gp140 (J408 and H445), unmodified SF162gp140 (P655 and N472) and recombinant gp120 (L714 and

1 L814). The co-receptor usage of each isolate is shown in parenthesis. The percent  
2 neutralization was calculated as described in Materials and Methods taking into  
3 consideration the non-specific neutralization recorded with sera collected from the  
4 same animals prior to the initiation of the immunization schedule. <sup>&</sup> (A): sera collected  
5 2 weeks following the DNA plus protein 'booster' immunization and (B) sera  
6 collected 2 weeks following the final protein 'booster' immunization of animals J408  
7 and H445. Values represent averages from two to three independent experiments. The  
8 susceptibility of these isolates to neutralization by 2F5 and 2G12 at 25 µg /ml of MAb  
9 is also presented. NT: Not evaluated.

10

11 Heterologous isolate-neutralization was not recorded (less than 50% inhibition of  
12 infection at 1:10 serum dilution) during the DNA-phase of immunization in macaques.

13 Two weeks following the DNA plus protein 'booster' immunization, sera collected  
14 from the two animals vaccinated with the modified  $\Delta V2gp140$  protein, neutralized  
15 some of the heterologous primary HIV-1 isolates tested (**Figure 10**). At the lowest  
16 serum dilution tested (1:10), and when non-specific neutralization recorded with pre-  
17 immunization sera was taken into consideration (see Materials and Methods for  
18 details), 80-90% inhibition of infection was only recorded with the ADA, 91US056  
19 and 92US714 isolates by J408 sera and with the ADA, 92US714 and 92US660 isolates  
20 with the H445 sera (**Figure 10** and Table 2). The cross-neutralizing activity of the sera  
21 collected from these two animals differed. For example, 92US660-infection was  
22 inhibited by 80% and 50%, by H445 and J408 sera, respectively. The serum cross-  
23 neutralizing activity decreased during the subsequent weeks of observation (**Figure**  
24 **10**). Sera collected 5 weeks following this DNA plus protein 'booster' immunization,  
25 had no cross-reactive neutralizing activity, even though potent neutralization of the  
26 SF162 and SF162 $\Delta V2$  isolates was still recorded.

1  
2 Despite the fact that following this DNA plus protein 'booster' immunization, the  
3 binding antibody titers in animals vaccinated with the unmodified immunogen were  
4 higher than those in animals vaccinated with the modified immunogen (Figure 8), the  
5 former sera failed to neutralize any of the heterologous isolates tested (Table 2) (i.e.,  
6 less than 50% specific neutralization was recorded). Thus, although in rabbits the  
7 unmodified immunogen was able to elicit (albeit much less efficiently than the  
8 modified immunogen) neutralizing antibodies against some heterologous primary  
9 HIV-1 isolates (Table 1), it failed to do so in rhesus macaques.

In parallel, the susceptibility was evaluated of the heterologous isolates to neutralization by sera collected from macaques that have been immunized with the recombinant SF2-derived gp120 protein. This protein was previously evaluated as a vaccine candidate and was ineffective in eliciting cross-reactive neutralizing antibodies, i.e., less than 50% neutralization at serum dilutions of 1:10 was recorded (Mascola, J. R., S. W. Snyder, O. S. Weislow, S. M. Belay, R. B. Belshe, D. H. Schwartz, M. L. Clements, R. Dolin, B. S. Graham, G. J. Gorse, M. C. Keefer, M. J. McElrath, M. C. Walker, K. F. Wagner, J. G. McNeil, F. E. McCutchan, D. S. Burke and the NIAID AIDS vaccine evaluation group. 1996. Immunization with envelope subunit vaccine products elicits neutralizing antibodies against laboratory-adapted but not primary isolates of human immunodeficiency virus type 1. *J. Infect. Dis.* 173:340-348). All the isolates tested here were not susceptible to neutralization by antibodies elicited by the SF2 gp120 protein (Table 2).

23

24 Second 'booster' immunization with the modified  $\Delta V2gp140$  protein: Although the

25 above results indicated that the modified  $\Delta V2gp140$  immunogen was indeed more

26 effective in eliciting cross-reactive neutralizing antibody responses than the

1 unmodified immunogen, these responses were weaker than those recorded against the  
2 parental SF162 isolate (**Figure 11A-B**). In an effort to further increase the potency and  
3 breath of these responses, an attempt was made to further 'boost' the antibody titers in  
4 animals H445 and J408 by immunizing them one additional time with the purified  
5 oligomeric  $\Delta V2gp140$  protein (this time in the absence of DNA-immunization).

6  
7 An increase in antibody-titers was indeed recorded following this protein 'boost', so  
8 that at their peak value (1:145,000 end-point ELISA titers) the titers were  
9 approximately 3 fold higher than those recorded during the first 'booster'  
10 immunization with DNA plus protein (**Figure 11A**). In parallel, a significant increase  
11 was found in the titer of neutralizing antibodies against the homologous SF162 $\Delta V2$   
12 and parental SF162 isolates (**Figure 11B**). No differences in the neutralizing potential  
13 of the sera collected 2 and 5 weeks following this last 'boost' were recorded, even  
14 though the binding antibody titers decreased significantly during the same period.  
15 Unexpectedly, however, the neutralizing potential of the same sera against most of the  
16 heterologous primary isolates tested generally decreased (Table 2). Thus, with the  
17 exception of the BZ167, 92US657 and ADA isolates, all the heterologous isolates  
18 tested were resistant to neutralization by sera collected 2 weeks following the second  
19 'boost'. Interestingly, although isolate 92US657 was resistant to neutralization by sera  
20 collected following the first boost, it became susceptible to neutralization by sera  
21 collected following the second boost.

22  
23 Generation of anti-V3 loop antibodies in Rhesus vaccinated with the modified  
24  $\Delta V2gp140$  immunogen: One explanation for the increase in neutralizing activity  
25 against the parental SF162 and homologous SF162 $\Delta V2$  viruses and the decrease in  
26 neutralizing activity against the heterologous isolates following the second 'booster'

1 immunization, is that multiple immunizations with the modified  $\Delta$ V2gp140 protein  
 2 increased the titer of antibodies directed against epitopes that are uniquely (or  
 3 predominantly) expressed on the SF162 and SF162 $\Delta$ V2 envelopes. It is conceivable  
 4 that multiple immunizations with the  $\Delta$ V2gp140 protein result in the generation of  
 5 high titers of anti-V3 loop antibodies. To determine the titer of such antibodies, a V3  
 6 loop peptide-based ELISA assays was used using the SF162/SF162 $\Delta$ V2-derived V3  
 7 loop (**Figure 12A-B**). This peptide was recognized by antibodies binding to both  
 8 linear (447D) (Conley, A. J., M. K. Gorny, J. A. Kessler, second, L. J. Boots, M.  
 9 Ossorio-Castro, S. Koenig, D. W. Lineberger, E. A. Emini, C. Williams, and S. Zolla-  
 10 Pazner. 1994. Neutralization of primary human immunodeficiency virus type 1  
 11 isolates by the broadly reactive anti-V3 monoclonal antibody, 447-52D. *J. Virol.*  
 12 68:6994-7000; Gorny, M. K., A. J. Conley, S. Karwowska, A. Buchbinder, J.-Y. Xu,  
 13 E. A. Emini, S. Koenig, and S. Zolla-Pazner. 1992. Neutralization of diverse human  
 14 immunodeficiency virus type 1 variants by an anti-V3 human monoclonal antibody. *J.*  
 15 *Virol.* 66:7538-7542) and conformational (391-95D) (Seligman, S. J., J. M. Binley, M.  
 16 K. Gorny, D. R. Burton, S. Zolla-Pazner, and K. A. Sokolowski. 1996.  
 17 Characterization by serial competition ELISAs of HIV-1 V3 loop epitopes recognized  
 18 by monoclonal antibodies. *Mol. Immunol.* 33:737-745) epitopes (**Figure 11A**).  
 19 Although anti-V3 loop antibodies were generated upon immunization of macaques  
 20 with the modified  $\Delta$ V2gp140 immunogen, their titers were much lower than those  
 21 against the entire envelope (**Figure 11B**). In addition, the second 'booster'  
 22 immunization did not increase the titer of anti-V3 loop antibodies. It should be noted,  
 23 however, that certain anti-V3 loop antibodies present in the serum of these animals  
 24 may not interact efficiently with the V3 loop peptide in an ELISA format, while they  
 25 may bind to their epitopes on the native envelope (Moore, J. P. 1993. The reactivities  
 26 of HIV-1+ human sera with solid-phase V3 loop peptides can be poor predictors of

1 their reactivities with V3 loops on native gp120 molecules. AIDS Res. Hum.  
2 Retroviruses 9:209-19). Additionally, the V3 loop peptide used here does not span the  
3 carboxy and amino termini of the V3 loop and the assay does not detect antibodies  
4 targeting these two regions. Thus, a more detailed examination of the epitope-  
5 specificity of the antibodies elicited by the modified  $\Delta$ V2gp140 immunogen is  
6 required.

8     Figures 13A and B demonstrate the heterologous immune response elicited by the  
9     immunogens of the invention, by the neutralization of HIV-1 viruses of different  
10    clades. Figure 13A shows the neutralization using serum from animals H445; Figure  
11    13B using serum from animal J408.

13 While the invention has been described and illustrated herein by references to the  
14 specific embodiments, various specific material, procedures and examples, it is  
15 understood that the invention is not restricted to the particular material combinations  
16 of material, and procedures selected for that purpose. Indeed, various modifications of  
17 the invention in addition to those described herein will become apparent to those  
18 skilled in the art from the foregoing description and the accompanying figures. Such  
19 modifications are intended to fall within the scope of the appended claims.

21 Various publications are cited herein, the disclosures of which are incorporated by  
22 reference in their entireties.